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Gene J. Yao
(Print Name)

(Signature)

Date: October 23, 2007



PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent application of:

Group: 1614

Friederike Hesse, et al.

Serial No.: 10/591,040

Filed: August 29, 2006

For: **METHOD FOR THE PURIFICATION OF AN N-TERMINAL FRAGMENT OF HEPATOCYTE GROWTH FACTOR**

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October 23, 2007

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P.O. Box 1450
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Dear Sir:

Attached please find one certified copies of the foreign applications from which priority is claimed for this case:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>
Europe	04004950.4	March 3, 2004

Respectfully submitted,

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Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

04004950.4

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 04004950.4
Demande no:

Anmeldetag:
Date of filing: 03.03.04
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Method for the purification of an N-terminal fragment of hepatocyte growth factor

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C07K14/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filling/Etats contractants désignés lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PL PT RO SE SI SK TR LI

03. März 2004

22389 EP-SR

**Method for the purification of an N-terminal fragment
of hepatocyte growth factor**

The invention relates to a method for the purification of the N-terminal four kringle-containing fragment of hepatocyte growth factor (NK4).

Background of the Invention

5 Hepatocyte growth factor (HGF/SF) is a polypeptide identified and purified by Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459. It was further found that hepatocyte growth factor is identical to scatter factor (SF), Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005. HGF is a glycoprotein involved in the development of a number of cellular phenotypes including proliferation, mitogenesis, formation of branching tubules and, in the case of tumor cells, invasion and metastasis. For a status review, see Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30.

10 Both rat HGF and human HGF have been sequenced and cloned (Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973; Nakamura, T., et al., Nature 342 (1989) 440-443; Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327; Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204; Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381).

15 It was further found that an HGF/SF fragment, termed NK4, consisting of the N-terminal hairpin domain and the four kringle domains of HGF/SF has pharmacological properties that are completely different from those of HGF/SF, and is an antagonist to the influence of HGF/SF on the motility and the invasion of colon cancer cells, and is, in addition, an angiogenesis inhibitor that suppresses tumor growth and metastasis (Parr, C., et al., Int. J. Cancer 85 (2000) 563-570; Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743; Date, K., et al., FEBS Lett. 420 (1997) 1-6; Date, K., et al., Oncogene 17 (1999) 3045-3054).

20 NK4 is prepared according to the state of the art (Date, K., et al., FEBS Lett. 420 (1997) 1-6) by recombinant expression of HGF cDNA in CHO cells and subsequent digestion with pancreatic elastase. Two other isoforms of HGF (NK1 and NK2) encoding the N-terminal domain and kringle 1, and the N-terminal domain and

kringles 1 and 2, respectively, were produced in E.coli via the inclusion body route (Stahl, S.J., Biochem. J. 326 (1997) 763-772). According to Stahl, naturation of NK1 or NK2 was performed in 100 mmol Tris/HCl pH 7.5 containing 2.5 M urea, 5 mM reduced glutathione and 1 mM oxidized glutathione. Purification was performed subsequently on a Superdex™ 75 column using also Tris buffer. The use of Tris buffer according to the state of the art during solubilization and naturation leads according to the investigations of the inventors to a considerable amount (of about 50%) of side-products which are identified by the inventors as consisting mainly of GSH-modified NK4.

10 Therefore this method is not useful for the recombinant production of NK4 in considerable amounts and sufficient purity.

Summary of the Invention

15 The invention provides a method for the production of NK4 by expression of a nucleic acid encoding said NK4 in a microbial host cell, isolation of inclusion bodies containing said NK4 in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK4 in the presence of GSH and GSSG, characterized in that solubilization and naturation are performed at pH 7-9 in phosphate buffered solution.

20 It was surprisingly found that the use of potassium phosphate buffer in a pH range between 7 and 9, preferably between pH 8 and 9, leads to a considerable improvement in yield and purity of NK4.

25 Preferably NK4 is dialyzed after naturation with phosphate buffer pH 7-9 for at least 24 hours. Purification is performed preferably by hydrophobic interaction chromatography in the presence of phosphate buffer pH 7-9, whereby the use of butyl- or phenyl sepharose as chromatographic material is especially preferred.

Detailed Description of the Invention

30 Human HGF is a disulfide-linked heterodimer, which can be cleaved in an α -subunit of 463 amino acids and a β -subunit of 234 amino acids, by cleavage between amino acids R494 and V495. The N-terminus of the α -chain is preceded

5 by 31 amino acids started with a methionine group. This segment includes a signal sequence of 31 amino acids. The α -chain starts at amino acid 32 and contains four kringle domains. The so-called "hairpin domain" consists of amino acids 70-96. The kringle 1 domain consists of amino acids 128-206. The kringle 2 domain consists of amino acids 211-288, the kringle 3 domain consists of amino acids 305-383, and the kringle 4 domain consists of amino acids 391-469 of the α -chain, approximately.

10 NK4 according to the invention consist preferably of aa 32-494 or a N-terminal fragment thereof (always beginning with aa32), the smallest fragment being aa 32-478. and the length of NK4 can vary within this range as long as its biological properties are not affected. In addition there exist variations of these sequences, 15 essentially not affecting the biological properties of NK4 (especially not affecting its activities antagonistic to HGF and its antiangiogenic activities), which variations are described, for example, in WO 93/23541. The activity of NK4 is measured by a scatter assay according to example 4.

15 NK4 can be produced recombinantly, either by the production of recombinant human HGF/SF and digestion with elastase (Date, K., FEBS Lett. 420 (1997) 1-6) or by recombinant expression of an NK4 encoding nucleic acid in appropriate host cells, as described below. NK4 glycoprotein has a molecular weight of about 57 kDa (52 kDa for the polypeptide part alone) and has the in vivo biological activity of 20 causing inhibition of tumor growth, angiogenesis and/or metastasis.

25 The NK4 polypeptides can be produced by recombinant means in prokaryotes. For expression in prokaryotic host cells, the nucleic acid is integrated into a suitable expression vector, according to methods familiar to a person skilled in the art. Such an expression vector preferably contains a regulatable/inducible promoter. The recombinant vector is then introduced for the expression into a suitable host cell such as, e.g., *E. coli* and the transformed cell is cultured under conditions which allow expression of the heterologous gene. After fermentation inclusion bodies containing denatured NK4 are isolated.

30 *Escherichia*, *Salmonella*, *Streptomyces* or *Bacillus* are for example suitable as prokaryotic host organisms. For the production of NK4 polypeptides prokaryotes are transformed in the usual manner with the vector which contains the DNA coding for NK4 and subsequently fermented in the usual manner. However

expression yield in *E. coli* using the original NK4 DNA sequence (GenBank M73239) is very low. Surprisingly it was found, that modification at least one of the codons of the DNA sequence of positions 33 to 36 (codon 33 encodes arginine, numbering according to M73239) results in an increase of expression yield of 20% 5 polypeptide or more. Therefore a further object of the invention is a method for the recombinant production of NK4 in prokaryotes by expression of a replicable expression vector containing DNA encoding NK4 characterized in that in said DNA at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 34, 35 and 36 is modified from AGG to CGT (position 33), 10 AAA to AAA(position 34), AGA to CGT (position 35), and/or AGA to CGT(position 36). It is further preferred that the codon for amino acid 32 is changed from encoding Gln to encoding Ser in order to improve splitting off N-terminal arginine.

15 Inclusion bodies are found in the cytoplasm as the gene to be expressed does not contain a signal sequence. These inclusion bodies are separated from other cell components, for example by centrifugation after cell lysis.

20 The inclusion bodies were solubilized by adding a denaturing agent like 6 M guanidinium hydrochloride or 8 M urea at pH 7-9 in phosphate buffer (preferably in a concentration of 0.1 – 1.0 M, e.g.0.4 M) preferably in the presence of DTT (Dithio-1,4-threitol). The solubilisate is diluted in phosphate buffer pH 7-9 in the presence of GSH/GSSG (preferably 2-20 mM, glutathion) and a denaturing agent in a non denaturing concentration (e.g. 2M guanidinium hydrochloride or 4 M urea) or preferably instead of guanidinium hydrochloride or urea, arginine in a concentration of about 0.3 to 1.0 M, preferably in a concentration of about 0.7M. 25 Renaturation is performed preferably at a temperature of about 4°C and for about 48 to 160 hours.

After naturation is terminated the solution was dialyzed preferably against phosphate buffer pH 7-9 (preferably in a concentration of 0.1 – 1.0 M, e.g. 0.3 M) for at least 24 hours, preferably for 24 – 120 hours.

30 NK4 polypeptide or fragments thereof can be purified after recombinant production and naturation of the water insoluble denatured polypeptide (inclusion bodies) according to the method of the invention preferably by chromatographic

5 methods, e.g. by affinity chromatography, hydrophobic interaction chromatography, immunoprecipitation, gel filtration, ion exchange chromatography, chromatofocussing, isoelectric focussing, selective precipitation, electrophoresis, or the like. It is preferred to purify NK4 polypeptides by hydrophobic interaction chromatography, preferably at pH 7-9, in the presence of phosphate buffer and/or preferably by the use of butyl- or phenyl sepharose.

10 The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 Renaturation kinetics, heparin column, detection at 280 nm

Figure 2 Renaturation efficacies

Description of the Sequences

15 **SEQ ID NO:1** DNA coding for NK4

SEQ ID NO:1 Polypeptide sequence of NK4

Example 1

Recombinant expression of NK4

20 The NK4 domain from amino acid position 32 to 478 of HGF was used for cloning and recombinant expression in *Escherichia coli*. The original DNA sequence used as source of DNA was described (database identifier "gb:M73239"). PCR was performed in order to amplify and concurrently modify the DNA coding for NK4 (Seq ID No:1). All methods were performed under standard conditions.

25 In comparison to the original DNA sequence of NK4, the following modifications were introduced:

- Elimination of the eukaryotic signal peptide sequence and fusion of the ATG start codon next to amino acid position 32 of NK4
- exchange of amino acid position 32 from Gln to Ser in order to improve homogeneity of the protein product (Met-free)
- 5 - modification of the DNA sequence of the codons of amino acids at position 33 (AGG to CGT), 35 (AGA to CGT), and 36 (AGA to CGT) in order to improve gene expression in E.coli.
- modification of the DNA sequence of codons at position 477 (ATA to ATC) and 478 (GTC to GTT) in order to facilitate insertion of PCR product into the vector
- 10 - introduction of two translational stop codons at positions 479 (TAA) and 480 (TGA), in order to stop the translation at a position equivalent to the end of NK4 protein domain.

15 The PCR-amplified DNA fragment was treated with restriction endonucleases NdeI and BanII and was ligated to the modified pQE vector (Qiagen) (elimination of His-tag as well as DHFR coding region), which was appropriately treated with NdeI and BanII. The elements of expression plasmid pQE-NK4-Ser (Plasmid size 4447 bp) are T5 promotor/ lac operator element, NK4 coding region, lambda to transcriptional termination region, *rrnB* T1 transcriptional termination region, ColE1 origin of replication and β -lactamase coding sequence.

20

25 The ligation reaction was used to transform E.coli competent cells, e.g. E. coli strain C600 harbouring expression helper plasmid pUBS520 (already patented and published elsewhere). E.coli colonies were isolated and were characterized with respect to restriction and sequence analysis of their plamsids. The selection of clones was done by analysis of the NK4 protein content after cultivation of recombinant cells in LB medium in the presence of appropriate antibiotics and after induction of the gene expression by addition of IPTG (1mM). The protein pattern of cell lysates were compared by PAGE. The recombinant E.coli clone showing the highest proportion of NK4 protein was selected for the production process. Fermentation was performed under standard conditions and inclusion bodies were isolated.

30

Example 2

Solubilization and renaturation using the optimized conditions.

5 Inclusion bodies were dissolved over night in a buffer containing 6 M guanidinium hydrochloride, 0.1 M potassium phosphate pH 8.5 (by titration with 10 M KOH), mM EDTA, 0.01 mM DTT. The concentration of the dissolved protein was determined by Biuret assay and finally adjusted to a concentration of 25 mg total protein / ml at room temperature .

10 This NK4-solubilisate was diluted to a concentration of 0.4 mg / ml in a buffer containing 0.7 M arginine, 0.1 M potassium phosphate pH 8.5 (by titration with conc. HCl), 10 mM GSH, 5 mM GSSG and 1 mM EDTA. This renaturation assay was incubated between 2 and 8 days at 4°C. The renaturation efficacy was measured by analytical affinity chromatography using an 1 ml Heparin Sepharose column (renaturation kinetics see Fig.1).

15 **Buffer conditions:**

Buffer A: 50 mM Tris pH 8.0
Buffer B: 50 mM Tris pH 8.0, 2 M NaCl

20 Gradient: 5-25% buffer B, 2 column volumes
 25-60% buffer B, 16 column volumes
 60-100% buffer B, 0.7 column volumes
 100% buffer B, 2 column volumes

25 After obtaining the maximal renaturation efficacy, the renaturation assay of 15 l volume was concentrated to 3 l using a tangential flow filtration unit (MW cut off: 10 kDa, Sartorius). It was subsequently dialyzed against 3 times 50 l buffer containing 0.3 M potassium phosphate at pH 8.0 for at least 3 x 24 hours, optimally for 5 days in total.

30 Purification was performed by heparin-sepharose chromatography (conditions see above). To the eluted material 1 M ammonium sulfate in 0.1 M potassium phosphate pH 8.0 was added and incubated at 4°C overnight. The sample was centrifuged and the supernatant was loaded on a phenyl sepharose column (150

ml). The column was washed with 1 column volume 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0.

Elution conditions:

Buffer A: 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0

5 Buffer B: 50 mM potassium phosphate pH 8.0, 40 % ethylene glycol
0-100 % buffer B, 20 column volumes

Example 3

Comparison of renaturation using potassium phosphate and Tris

10 Renaturation conditions were compared using potassium phosphate or TRIS at pH 7.5 and pH 8.5 (both titrated with conc. HCl) as buffering reagents. The solubilization and renaturation conditions were as described in example 2, but with 0.1 M TRIS or 0.1 M potassium phosphate in the renaturation buffer. The dialysis was also performed as described in example 2, but in 0.1 M TRIS or 0.1 M 15 potassium phosphate. Potassium phosphate buffer (K-P) led to significantly higher renaturation efficacies as TRIS buffer, measured as amount of active NK4 by scatter assay (see Fig. 2).

Example 4

Determination of activity

20 a) **Scatter assay**

MDCK cells were subconfluently grown in tissue culture plates. Cells were treated with HGF (10 ng/ml) or with combinations of HGF and NK4. In these experiments the HGF-induced cell scattering was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

25 b) **Proliferation assay**

Inhibition of the mitogenic activity of HGF by NK4 was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture as described in Nakamura, T., et al., Nature 342 (1989) 440-443. In these experiments the HGF-

induced cell proliferation was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

c) **Invasion assay**

5 In this assay the invasive potential of tumor cells is analyzed. The assay was done essentially as described in Albini, A., et al., Cancer Res. 47 (1987) 3239-3245, using HT115 cells. Again, HGF-induced (10 ng/ml) cell invasion could be inhibited by a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

Example 5

10 **Activity in vivo**

Model: Lewis Lung Carcinoma nude mouse tumor model
1 x 10⁶ lewis lung carcinoma cells were s.c. implanted into male nude mice (BALB/c nu/nu).

15 Treatment: After 4 days, one application daily of pegylated NK4 over a period of 2-4 weeks

Dose: 1000 µg/mouse/day
300 µg/mouse/day
100 µg/mouse/day
placebo

20 Result: Treatment with NK4 shows a dose dependent suppression of primary tumor growth and metastasis, whereas no effect is seen in placebo treated groups.

List of References

Albini, A., et al., *Cancer Res.* 47 (1987) 3239-3245
Date, K., et al., *FEBS Lett.* 420 (1997) 1-6
Date, K., et al., *Oncogene* 17 (1989) 3045-3054
5 Kuba, K., et al., *Cancer Res.* 60 (2000) 6737-6743
Miyazawa, K. et al., *Biochem. Biophys. Res. Comm.* 163 (1989) 967-973
Nakamura, T., et al., *Biochem. Biophys. Res. Commun.* 22 (1984) 1450-1459
Nakamura, T., et al., *Nature* 342 (1989) 440-443
Okajima, A., et al., *Eur. J. Biochem.* 193 (1990) 375-381
10 Parr, C., et al., *Int. J. Cancer* 85 (2000) 563-570
Seki, T., et al., *Biochem. and Biophys. Res. Comm.* 172 (1990) 321-327
Stahl, S.J., *Biochem. J.* 326 (1997) 763-772
Stuart, K.A., et al., *Int. J. Exp. Pathol.* 81 (2000) 17-30
Tashiro, K., et al., *Proc. Natl. Acad. Sci. USA* 87 (1990) 3200-3204
15 U.S. Patent No. 5,977,310
Weidner, K.M., et al., *Proc. Natl. Acad. Sci. USA* 88 (1991) 7001-7005
WO 93/23541

03. März 2004

Patent Claims

1. Method for the production of a N-terminal four kringle-containing fragment of hepatocyte growth factor (NK4) by expression of a nucleic acid encoding said NK4 in a microbial host cell, isolating of inclusion bodies containing said NK4 in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK4, characterized in that solubilization and naturation are performed at pH 7-9 in phosphate buffered solution.
5
2. Method according to claim 1, wherein, after naturation, NK4 is dialyzed with phosphate buffer pH 7-9 for at least 24 h.
- 10 3. Method according to claim 1 or 2, characterized in that NK4 is purified after naturation by hydrophobic interaction chromatography in the presence of phosphate buffer at pH 7-9.
4. Method according to claim 3, characterized in that chromatography is performed on butyl- or phenyl sepharose.

03. März 2004

Abstract

A method for the production of the N-terminal four kringle-containing fragment of hepatocyte growth factor (NK4) by expression of a nucleic acid encoding said NK4 in a microbial host cell, isolating of inclusion bodies containing said NK4 in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK4, characterized in that solubilization and naturation are performed at pH 7-9 in phosphate buffered solution, provides NK4 in high purity and high yield.

03. März 2004

SEQUENCE LISTING

<110> F. Hoffmann-La Roche AG

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Glu His Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu
130 135 140

Asn Tyr Cys Arg Asn Pro Arg Gly Glu Gly Gly Pro Trp Cys Phe
145 150 155 160

Thr Ser Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys
165 170 175

Ser Glu Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Leu
180 185 190

Met Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln
195 200 205

Thr Pro His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly
210 215 220

Phe Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp
225 230 235 240

Cys Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys
245 250 255

Thr Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr
260 265 270

Thr Glu Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn
275 280 285

Thr Ile Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro
290 295 300

His Glu His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg
305 310 315 320

Glu Asn Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe
325 330 335

Thr Thr Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn
340 345 350

Cys Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn
355 360 365

Tyr Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met
370 375 380

Trp Asp Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro
385 390 395 400

Asp Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Asp
405 410 415

Ala His Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp
420 425 430

Tyr Cys Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val
435 440 445

03. März 2004

Fig. 1

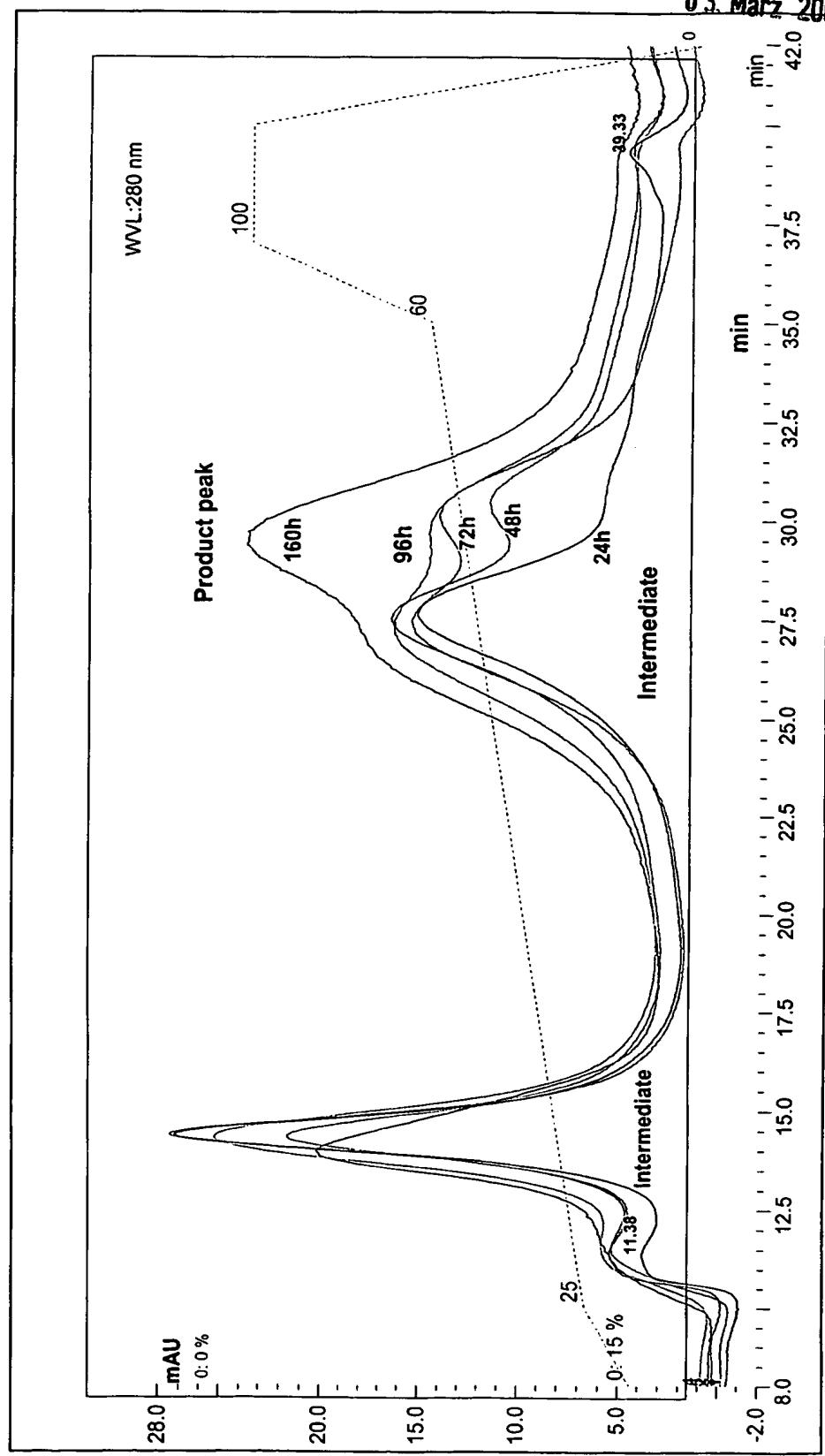


Fig. 2

